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Individual filamentous phage imaged by electron holography

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Abstract An in-line electron hologram of an individual f1.K phage was recorded with a purpose-built low energy electron point source (LEEPS) microscope. Cryo-microscopic methods were employed to prepare the specimen so that a single phage could be presented to the coherent low energy electrons: An aqueous phage suspension was applied to a thin carbon membrane with micro-machined slits. The membrane was rapidly cooled to freeze the remaining water as an amorphous ice sheet, which was then sublimated at low temperatures and pressures to leave individual free-standing phages suspended across slits. An image of a phage particle, depicted as the amplitude of the object wave, was reconstructed numerically from a digitized record of the hologram, obtained using 88 eV coherent electrons. The reconstructed image shows a single phage suspended across a slit in a supporting carbon membrane, magnified by a factor of 100,000. The width and shape in the reconstructed image compared well with a TEM image of the same filament. It is thus possible to

record and reconstruct electron holograms of an individual phage. The challenge now is to improve the resolution of reconstructed images obtained by this method and to extend these structural studies to other biological molecules.

Keywords Holography · Low energy electron microscopy · Single molecule

Introduction

The concept of in-line electron holography was originally proposed by Gabor (1948) to circumvent lens aberrations in the electron microscope. A practical demonstration of his original idea was made using a low energy electron point source (LEEPS) microscope, shown schematically in Fig. 1. This instrument has been used to obtain in-line electron holograms of single carbon filaments (Fink et al. 1990) and DNA (Fink et al. 1997), demonstrating the potential of LEEPS microscopy for imaging individual biological molecules.

A major advantage of using low energy (up to 200 eV) electrons to image biological molecules is that the amount of radiation damage is several orders of magnitude less than in conventional transmission electron microscopy (TEM), as has recently been shown (Germann et al. 2010). In order to obtain high-resolution images with conventional TEM, the high radiation damage necessitates averaging over many molecules, with a corresponding limitation in positional accuracy. In addition, low energy electrons are more strongly scattered than high energy electrons by the lighter elements that make up biological molecules, enabling high contrast holograms to be acquired.

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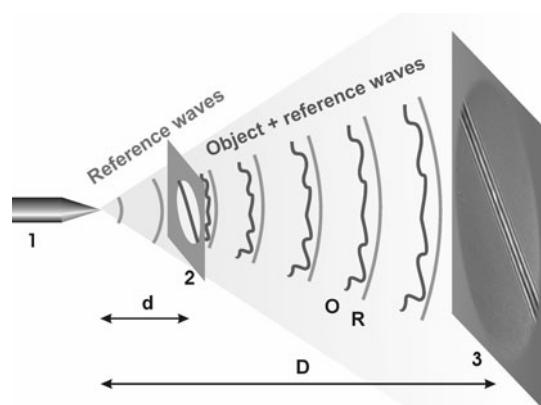


Fig. 1 Schematic diagram of a LEEPS microscope: an electron point source (1) is held at a negative voltage with respect to a specimen filament supported across a hole in a carbon film (2) located at a microscopic distance (d) from the source. The film supports the macromolecule and serves as an extractor electrode. A spherical reference electron wave (R) emanates from the source and expands as it approaches the specimen where it is scattered, forming the object wave (O). A hologram is formed on a screen (3), located at a macroscopic distance (D) from the specimen, due to interference between O and R

The holographic technique provides phase and amplitude information of the object so that, in principle, a three-dimensional structure can be recovered. As shown schematically in Fig. 1, a hologram is formed by interference between a coherent reference wave and an object wave that is scattered by the specimen.

In order to obtain electron holograms of a single molecule, the molecule must be placed in front of the coherent electron source so that part of the beam gets scattered by the molecule forming the object beam, and the other part passes the object unchanged, thus forming the reference beam. Ideally, the molecule would be free standing in vacuum, but in practice a support structure is required. One approach is to use a filamentous object that has been suspended across a slit in a supporting film.

We used a filamentous bacteriophage of the inovirus type, exemplified by fd, f1, and M13 (van Houten et al. 2006), which is a flexible filament about 1 μm long and 6.5 nm in diameter. The particular phage chosen for these experiments is a mutant of phage f1, called f1.K, which differs from the wild-type f1 phage by an insertion of four amino acids (Ala-Lys-Ala-Ser) behind amino acid 3 of the major coat protein g8p (van Houten et al. 2006), whose sequence on the phage thus begins $\text{NH}_2\text{-Ala-Glu-Gly-Ala-Lys-Ala-Ser-Asp-Asp-Pro}$. In future experiments, this phage may be used as a convenient and particularly robust scaffold to support other molecules in the electron beam because it can be derivatized at the exposed engineered lysine residue in g8p.

Here we describe how a specimen of f1.K was prepared so that a low energy in-line electron hologram of an individual phage could be obtained. The recorded hologram was numerically reconstructed and compared with a TEM image of the phage.

Materials and methods

Specimen preparation

Phages were purified and propagated in XL1-Blue *E. coli* cells (Russel et al. 2004) and isolated by cesium chloride density centrifugation. The phage concentration of 5×10^{11} phages/ml was determined by UV absorption at 269 nm. Carbon TEM grids were prepared by milling arrays of 400 nm \times 2 μm slits using a focused beam of gallium ions (Orloff et al. 2003). As shown in Fig. 2a, each slit was uniquely labeled so that slits of interest could be easily found in the microscope. Before applying a droplet of phage suspension, the surface of the carbon film was made hydrophilic by exposing it to a radio-frequency glow discharge in residual air for several seconds. A droplet of phages suspended in 20 mM phosphate buffer at pH 7.8 was then applied to the grid. The droplet was removed after 60 s with a filter paper, and the grid was rinsed twice by applying and removing a droplet of water. The grid with a thin layer of remaining water was rapidly plunged into liquefied ethane held at a temperature of 100 K in a purpose-built freeze-drying chamber.

The grid was kept at low temperatures while the gas in the drying chamber was pumped out using rotary and turbo pumps. The temperature was then allowed to increase to 250 K over 7 h to allow the ice to sublime, leaving behind phages on the carbon film (Dubochet et al. 1988).

As shown in Fig. 2b, c, some filaments remained across slits in the film. The use of freeze-drying prevented the rearrangement of phages around the edges of slits, which occurred when the phages were dried at room temperature and ambient pressure.

The TEM micrograph at higher magnification, Fig. 2c, shows an unstained filament suspended across one of the slits. The average width of the object in this image was 7 nm, which is in good agreement with the published width of f1 phages (Marvin 1998) and suggests that this is an image of a single phage. The variation in the width of the phage along part of its length is probably due to contamination. It is interesting to note that the unsupported molecule could be imaged with 100 keV electrons. However, we mention in passing that an unsupported double-stranded DNA has also previously been imaged with 100 keV electrons (Fujiyoshi and Uyeda 1981).

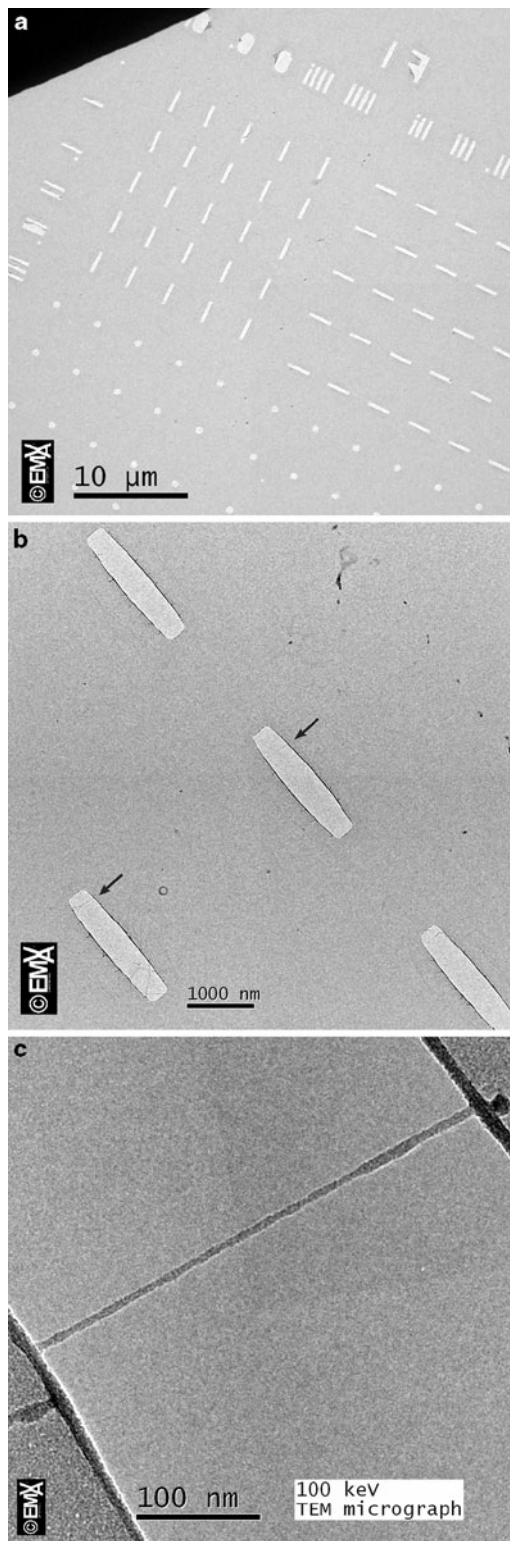


Fig. 2 TEM micrographs (100 keV) of a carbon film showing **a** micro-machined slits at low magnification to show the addressing system; **b** unstained phage specimen after freeze-drying (arrows show the location of filaments across slits); **c** close-up of phage suspended across the central slit of Fig. 2b

Holographic imaging and image reconstruction

The grid was then placed in the LEEPS microscope, and the electron point source, at a bias voltage of -88 V, was brought within a distance of several microns from the carbon support membrane. Using the addressing system on the grid, the phage shown in Fig. 2c was relocated, and a hologram of it was recorded.

The hologram was formed on a region of the detector screen approximately 7×7 mm, which was optically magnified before being digitally recorded with a CCD camera. The hologram of the phage, Fig. 3a, consists of several fringes extending across the width of the slit in the carbon film, the edges of which can also be seen in the hologram. The center of a bright Fresnel interference fringe near the edge of the carbon film indicates the location of the edge of the slit (Spence and Qian 1994). A line profile perpendicular to the fringes, Fig. 3b, shows the variation in intensity across the fringes.

An image of the filament was numerically reconstructed from the digitized electron holograms using software developed in-house. Before applying the reconstruction procedure, the hologram was normalized to give the relationship $\chi(\vec{K}) = (I - I_0)/I_0$, where $\chi(\vec{K})$ is the hologram function, \vec{K} is the emission vector, I is the intensity of the hologram, and I_0 is the intensity of the background. The result of this normalization is shown in Fig. 3c. Numerical reconstruction was done by applying the following transformation to the normalized holograms (Barton 1988):

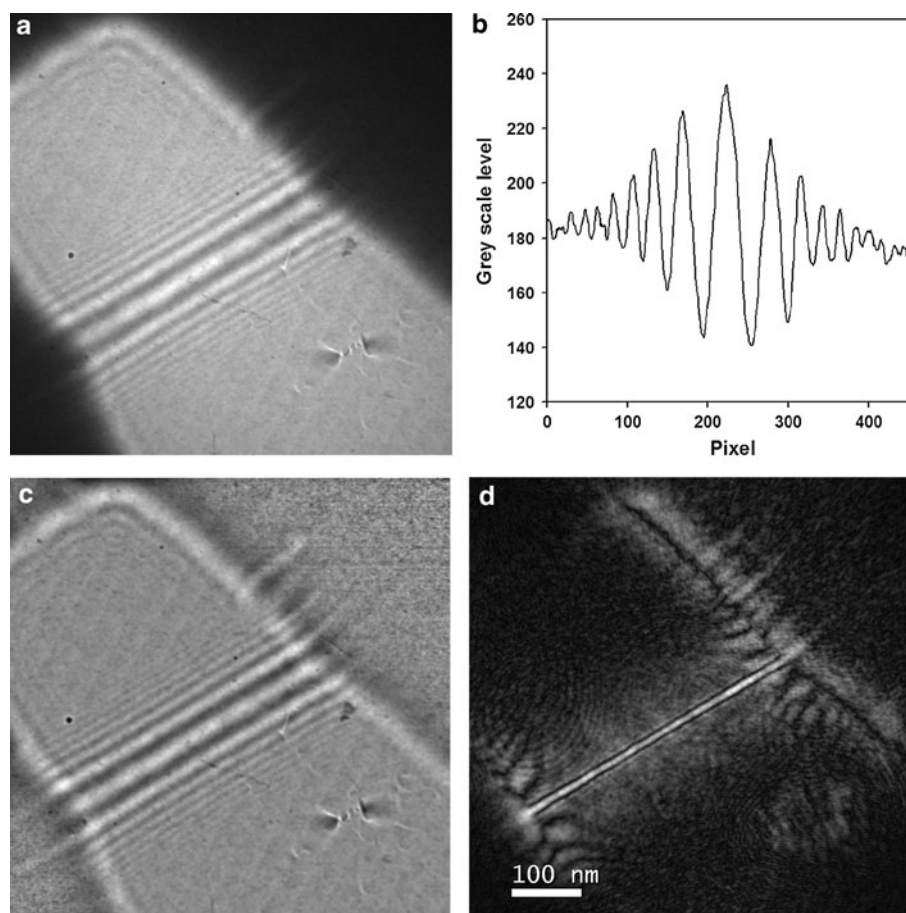
$$U(\vec{r}) = \frac{1}{2\pi R^2} \int_S \chi(\vec{K}) \exp(ik\vec{r}\vec{K}) d\sigma. \quad (1)$$

Here, $U(\vec{r})$ is the reconstructed complex field. The reconstruction, Fig. 3d, shows the amplitude of the reconstructed complex function $U(\vec{r})$ obtained for an optimal source-object distance. In the image, the intensity (gray scale level) is proportional to the reconstructed amplitude.

The size and shape of the filament suspended across the slit shown in the reconstruction of Fig. 3d are in good agreement with the TEM image of the phage in Fig. 2c. The light halo around the image of the phage is interpreted as the so-called twin image. In the object plane, the out-of-focus twin image is superimposed onto the object (Gabor 1949). The stub projecting into the slit is faintly visible in the reconstruction but is partially obscured by the twin image.

This work demonstrates that in-line electron holography can be used to image biological macromolecules. It remains to improve the ability to detect high order inter-

Fig. 3 **a** In-line low energy electron hologram of the f1.k phage shown in Fig. 2c. The source bias voltage and current used to obtain the hologram were 88 V and 97 nA, respectively. The estimated source-sample distance was 7.4 μm . **b** Intensity profile at the center of the hologram along a line perpendicular to the fringes. **c** Hologram after normalization (see main text). **d** Numerical reconstruction of the hologram (c). The source-specimen distance used to obtain the reconstruction was 6 μm



ference fringes so that the reconstructed image can be obtained at a resolution that allows protein subdomain structures to be visible. While beam damage is known to limit the resolution of images of single nonsymmetrical particles obtainable with conventional electron microscopy to about 10 Å (Frank 2002), in low energy electron holography the permissible dose is several orders of magnitude larger and thus imposes no limitations if it comes to image just one single biological molecule (Germann et al. 2010).

Conclusions

We report the recording and reconstruction of an electron hologram of an individual filamentous phage. Compared with previous experiments on imaging carbon filaments and DNA by low energy electron holography, we have made some important progress:

Plunge-freezing and freeze-drying were used to preserve the morphology of the biological sample. This technique had not been previously applied to imaging by low-energy electron holography.

A single phage was suspended across micro-machined slits in a carbon TEM grid. The dimensions of the objects in the TEM image show the single phage to be spanning the slits. The use of a novel micro-machined addressing system allowed for a direct comparison of images obtained with a LEEPS microscope and TEM.

A high contrast electron hologram (Fig. 3b) of an individual phage was obtained. The amplitude of the object wave was reconstructed numerically from this hologram (Fig. 3d). Although the reconstructed image doesn't provide any useful structural information, it has a close resemblance to the TEM image of the same filament, confirming the reconstruction to be a true representation of the original object.

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